EXHIBIT 6

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Antibodies of Predetermined Specificity in Biology and Medicine

RICHARD ALAN LERNER

Department of Molecular Biology,
Research Institute of Scripps Clinic,
La Jolla, California

I. Introduction

The diversity and exquisite specificity of antibodies has captured the imagination of scientists since the time of Erlich. And since it is possible to make use of the vast antibody repertoire to make antibodies specific for virtually any protein, it might have been reasonable to ask what more does one want? As is usually true in science, we are learning that there is indeed much, much more. The first new major advance was the development by Kohler and Milstein (1975) of the hybridoma technology. This development allowed the purification of individual specificities from the set of responses that an animal makes upon being presented with a collection of epitopes in one or even a mixture of molecules. Thus in one elegant stroke we were capable of having unlimited quantities of antibodies reactive with a single epi-

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tope. But, what epitope? It turns out that even with monoclonal antibodies this is a difficult question to answer and one which can only be approached by an often complex protocol of biochemical or genetic experiments. The basic problem, of course, is that the inducing immunogen is complex and the sorting out of specificities is a retrospective exercise.

Recently, it has become clear that antibodies to most regions of a protein can be induced by immunizing with short synthetic peptides. This advance portends a change in the way we generate and think about antibodies from a retrospective to a prospective science. Indeed, the synthetic immunogenic technology has already had impact on diverse areas of biology and medicine.

First, since the region in the protein with which antipeptide antibodies react is known in advance to the experimenter, they can be said to be of predetermined specificity. This has become useful in the search for protein products predicted from the nucleic acid sequence of newly described nucleic acid clones. Also, the predetermined specificity of these antibodies allows protein chemists and cell biologists to carry out precise structure–function experiments, and to orient proteins in cellular compartments and subcellular organelles.

Second, protein reactive antipeptide antibodies are being used to study the chemistry and structure of antigen–antibody union. A subset of the general problem of the chemistry of antigen–antibody union is the chemistry of virus neutralization. The synthetic immunogen technology has already led us a long way toward answers in this area.

Third, and perhaps in the long run the most important, the frequency with which protein-reactive antipeptide antibodies can be generated has led to a conceptual and experimental merger between the antibody and protein dynamics problems.

The details surrounding these three broad issues form the subject of this review. The search of the literature for this article ended in December of 1983. As for reviews in any area of rapidly progressing science, I must apologize in advance for not citing papers which have escaped my attention.

II. The Nature of Immunogen Determinants of Intact Proteins

As we will see, the nature of protein reactive antibodies induced by small synthetic peptides is probably very different from the antibodies induced by intact proteins. Thus, this section will be brief and will serve only to orient the reader to certain key conceptional issues. The issue of protein immunogenicity has been previously authoritatively

reviewed by Crumpton (1974) and more recently by a consortium of experts in the field (Benjamin *et al.*, 1984).

Before beginning the discussion we need to deal with one definition which concerns the difference between antigenicity and immunogenicity. Immunogenicity refers to the ability of a substance to induce an antibody, whereas antigenicity simply refers to the ability to be recognized by an antibody. Sometimes, when considering proteins, these terms blur. However, the differences can be crystalized when considering the polysaccharide coat of the pneumococcus. If a rabbit is immunized with intact pneumococcal bacteria an antibody to the polysaccharide coat is made. Thus under these circumstances, the polysaccharide is immunogenic (will induce antibody) and antigenic (will bind to antibody). If the experiment is repeated with the isolated, purified, polysaccharide no antibody is induced. Thus the purified polysaccharide is not immunogenic but is still antigenic in that it will react with antibody induced by the intact organism. This distinction will become important when antipeptide antibodies are considered since peptides can induce protein reactive antibodies which cannot be induced by immunizing with the intact protein. Thus, there are regions of a protein which are antigenic but not immunogenic. Indeed, as we will see, this is one of the most powerful aspects of the synthetic immunogen technology.

For as long as the antibody problem has existed, there have been two obvious routes to a solution. One could study either the antigen or the antibody.

To understand the way that studies proceeded, one needs to reflect on the scientific climate of immunology in the late 1940s and early 1950s. There were a number of things we knew (reviewed by Edelman, 1973). Working with haptenes, Landsteiner had provided evidence for molecul r complimentarity in antigen–antibody union (Landsteiner, 1936), and there was evidence that most antibodies were multivalent (Marrack, 1938). Tiselius (1937) had shown that antibodies were heterogeneous in charge and others had demonstrated heterogeneity in their binding constants (Karush, 1962). But little was known about the structure of antibodies and it was not until the late 1950s that the structure of immunoglobulins began to be revealed (Edelman, 1973; Porter, 1973).

Considering this lack of knowledge about the chemistry of antibodies it is no surprise that prior to 1950 studies in immunology centered around the antigens. In general, the approach which most workers took was to make antibodies to an intact protein and then determine the effect of proteolysis or denaturation on its antigenicity. As we will

detail in this section, these studies were to lead to two general conclusions about the antigenicity and immunogenicity of proteins. These were that only a limited portion of an intact protein is immunogenic and that antigenic determinants of proteins were most often constructed from amino acids distant from each other in the sequence but brought into proximity by the tertiary folding of the protein chain. These determinants were often called "conformational" or "discontinuous" and more recently have been referred to as assembled topographical determinants (Benjamin *et al.*, 1984).

As he had done for simple haptenes, Landsteiner was to lead the way into the study of the antigenicity of proteins. Writing in 1936, Landsteiner was to thus comment on the serological specificity of proteins, "In view of the imperfect state of protein chemistry, it is not surprising that neither the observations on natural antigens nor those on modified proteins suffice definitely to interpret the specificity of protein reactions in terms of chemical structure. Progress in this direction may come from pursuing the investigation of protein split products." He continues, "Clearly the highl selective action of the immune sera precludes specificity being determined by simple structures as single amino acids, and even reacting groups composed of di- or tripeptides could not furnish a sufficient number of combinations. Irrespective, therefore, of any particular hypothesis concerning their constitution, the specificity of proteins must be referable to complicated structures—possibly multiple, like groups in one molecule—or to several groupings whose affinities have to be satisfied before a visible reaction can occur, in which event the spatial arrangement of the reacting groups may be significant." Based on these thoughts, a genre of experiments designed to understand the nature of antigenicity was born. If the essential nature of an antigenic determinant is complex, what is the basis of this complexity?

The most telling experiments were those carried out on globular proteins where it was soon learned that upon denaturation their reactivity with homologous antibodies was almost completely eliminated (reviewed, Crumpton, 1974). Brown *et al.* (1959) showed that oxidation of all four of the disulfide bonds of ribonuclease eliminated its reactivity with an antibody against the native molecule. This initial finding was confirmed by many others using a variety of proteins (Freedman and Sela, 1966; Neumann *et al.*, 1967; Shapira and Arnon, 1969; Arnon and Neurath, 1970; Young and Leung, 1970; Goetzl and Peters, 1972).

Further evidence for the role of protein conformation in antigenicity came from the reciprocal experiments in which the ability of antibodies prepared against denatured proteins to react with the native protein was studied Again, the importance of conformation for antigenicity was demonstrated. Antibodies to denatured ribonuclease (Brown, 1962), lysozyme (Young and Leung, 1970; Arnon and Maron, 1971), or bovine serum albumin (Habeeb and Atassi, 1971) reacted only poorly if at all with the native molecule. The totality of experiments on denatured molecules was to be summarized in 1975 by Reichlin (Reichlin, 1975): "The essential meaning of these observations is that the native and fully denatured forms of proteins are for the most part entirely different structures from the immunological point of view."

The other constellation of experiments designed to understand the complexity of protein antigens were the fragmentation studies. As for denatured proteins, the general conclusion of such studies was that enzymatic or chemical digestion of proteins is accompanied by greatly diminished antigenicity (reviewed, Crumpton, 1974). For example Nisonoff *et al.* (1970) were not successful in obtaining immunologically reactive fragments of ribonuclease.

II The Loop of Lysozyme Experiments

The experiments carried out in the late 1960s and early 1970s by Arnon, Sela, and Anfinsen and their collaborators on residues 64–82 (the "loop") of lysozyme were of singular influence in crystallizing the concept that the antigenic determinants of proteins were conformation dependent (Arnon *et al.*, 1971). Thus, these experiments are discussed separately here. Based on the X-ray crystallographic data of Canfield and Liu (1965), Arnon and Sela (1969) noted that residues 64–80 of lysozyme formed a loop held together at the neck as it were, by a disulfide bond between Cys 64 and Cys 80. Thus, this structure offered a crisp experimental system to test the postulate that the majority of antigenic determinants of globular proteins were dependent on complex conformations. The question concerned the comparative immunogenicity and antigenicity of the open (disulfide bonds reduced and carboxymethylated) versus the closed (intrachain disulfide bonds intact) loop. The results showed that antibodies to intact lysozyme as well as antibodies to the closed loop bound to the closed loop, whereas oxidation of the disulfide bridge of the isolated loop resulted in a molecular species with a drastic decrease in ability to bind either of these antibodies (Arnon and Sela, 1969; Maron *et al.*, 1971; Arnon and Maron, 1971). In the broad sense these experiments impacted on two fronts. First, by direct experimentation, including chemical syn-

thesis of antigenic determinants of intact proteins, they were to again show the conformational nature of antigenic determinants of proteins. But, of more significance to this review, the experiments suggested that in order to synthesize immunogens which mimicked those of intact proteins, one needed to build complex conformations, a possibility difficult at best and only feasible where the X-ray structure of the protein had been determined.

IV. On the Number of Antigenic Determinants in Proteins

Given that antigenic determinants of globular proteins were of the conformational type, the next question considered was how many of these there were for a given protein. The general conclusions were that the number of antigenic sites was limited and on the *average* there was about one site for each 5000 daltons of protein (reviewed, Crumpton 1975; Benjamin *et al.*, 1984). In fact some authors argued that the complete antigenic structures of myoglobin (Atassi, 1975) and lysozyme (Atassi, 1978) were known. In myoglobulin, for example, Atassi suggested that the complete "immunological anatomy" consisted of five antigenic sites comprising residues 15–22, 56–62, 94–99, 113–119, and 145–151. In lysozyme Atassi and Lee (1978) proposed only three sites formed respectively by the discontinuous residues 5,7,13,14; 33,34,113,114; and 62,87,89,93,96,97. Recently the notion that a few sites determined the entire antigenicity of a protein has come under considerable attack (reviewed, Benjamin *et al.*, 1984). In a particularly telling series of experiments, Ibrahimi *et al.* (1979) and White *et al.* (1978) studied lysozymes with evolutionary substitutions at positions outside the antigenic sites proposed by Atassi and colleagues. Such lysozymes were shown to be antigenically distinct, thus showing that there were more antigenic sites than previously suggested. It is, of course, still possible that a change in the molecule at one site could alter one of the original determinant sites. The suggestion that there are sites additional to those previously proposed (Atassi, 1978) was given further support by the detailed studies of Smith-Gill and colleagues (1982). These investigators made a monoclonal antibody to chicken lysozyme C. This antibody reacted completely with lysozymes of seven different species of galliform birds, partially with two other galliform species and not all with duck lysozyme. The site of antibody binding was determined by comparing the amino acid sequences of these different lysozymes (again assuming that antigenic changes due to substitution of amino acids during evolution do not result from long-range effects) and by showing that Biebrick Scarlet, a dye which binds to the catalytic site of lysozyme,

inhibits the binding of antibody to the enzyme. The antibody binding site was postulated to involve the Arg 68–Arg 45 complex and extend into the cleft between Arg 45 and Arg 114 giving an overall dimension for the site of at least 13 × 6 × 15 Å. The important point relative to the present discussion is that this site is outside those defined by Atassi and Lee (1978). Perhaps the lesson in all this is that it is unrealistic to assume that one can define the complete immunogenicity and antigenicity of a protein on the basis of a single antiserum and that what is immunogenic may depend as much on the mode of presentation of the antigen and on the species immunized as the protein structure itself.

From the point of view of this review, however, the important point is that together, the two general assumptions that antigenic determinants were discontinuous and relatively few in number did not bode well for a general technology in which antibodies to any region of a protein could be generated.

V. Antibodies of Predetermined Specificity

We now come to the main substance of the review which is the concepts and applications which surround antipeptide antibodies of predetermined specificity. At the outset it is necessary to understand a generality which will become refined as the arguments proceed. This is that these antibodies are in principle very different from those made against intact proteins. Antibodies induced by intact proteins are generated by largely ordered arrays of atoms and then, depending on the experiment, tested against an ordered or experimentally disordered protein target. Conversely, antibodies of predetermined specificity are made against a disordered array of atoms and tested against an always more ordered protein target.

In 1980, signals began to appear which suggested the possibility for a new technology. Upon completion of the nucleotide sequence of the replication competent murine Maloney leukemia virus we were faced with an open reading frame which predicted a protein which we did not understand. The reading frame was part of the envelope gene and extended to the 3′-most end of the coding region of the gene and thus predicted an unexplained C-terminal segment of a protein (Sutcliffe *et al.*, 1980). We chemically synthesized a peptide predicted from the last nucleotide of the reading frame and made antibodies to it. The antibody precipitated two proteins from infected and transferred cells which corresponded to the envelope precursor protein, gp85, and a precursor to the membrane anchor protein, p15E, which is now called pre-15E (Green *et al.*, 1981). As it turned out, the reason that the predicted protein sequence did correspond with what we previously

knew about biochemistry of p15E is that during viral budding this protein undergoes a maturation step which involves cleavage and removal of a C-terminal fragment. Accordingly, the antibody which is site specific for the C-terminus detects the precursor but not the product molecule (Green *et al.*, 1981). Thus the antibody solved two problems at once each of which was dependent on its predetermined nature. The product of an open reading frame was established and a small site on a protein was specifically followed during a biological process. Walter *et al.* (1980) made antibodies to synthetic peptides corresponding to the carboxy- and amino-terminal regions of the simian virus 40 large tumor antigen. These antisera reacted appropriately with the intact large T antigen. These two studies signaled the possibility of a new technology, but there was a large conceptual hurdle to pass. This had to do with the fact that both studies involved the termini of proteins which often may be disordered and perhaps more easily mimicked by short peptides. Indeed, there had been some success in the past using the termini of proteins as immunogens (Anderer and Schlumberger, 1965; Arnon *et al.*, 1976) but little attempt was made to move beyond this. Thus, because of the argument about the ends of molecules and the general tenure of past arguments (see above) indicating that generally one needed to construct conformations to generate protein reactive antibodies, there was little certainty that the technology could be used broadly or in particular extend beyond the termini of proteins. If the argument about the termini of proteins held, synthetic immunogens would be of limited use and certainly not a general way of inducing site-specific immunological reagents for the study of proteins. However, Green and her colleagues carried out an experiment which suggested one could use synthetic immunogens to generate antibodies of predetermined specificity which were reactive with virtually any region of a protein (Green *et al.*, 1982). They used the influenza virus hemagglutinin as a test object because the complete nucleotide sequence of its gene was available (Min Jou *et al.*, 1980) and its crystallographic structure was known at high resolution (Wilson *et al.*, 1981).

A series of peptides covering 75% of the HA1 chain were chemically synthesized and antibodies were made to each. Antibodies to almost all (18 of 20) peptides reacted with the intact molecule (Green *et al.*, 1982). Since in its folded state the HA1 molecule displays a number of secondary structures including α-helix, extended chains, and B-

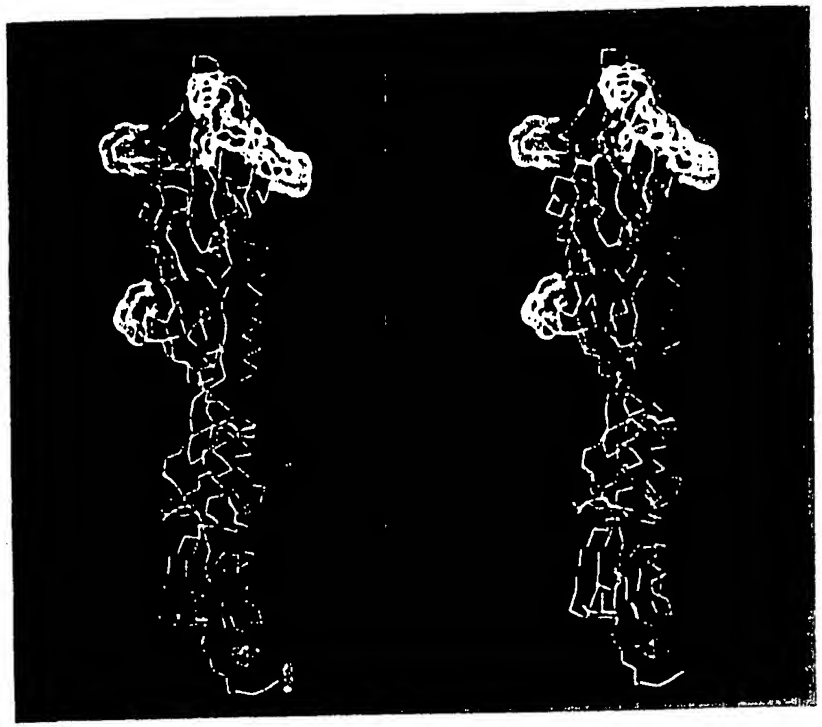

FIG. 1A.

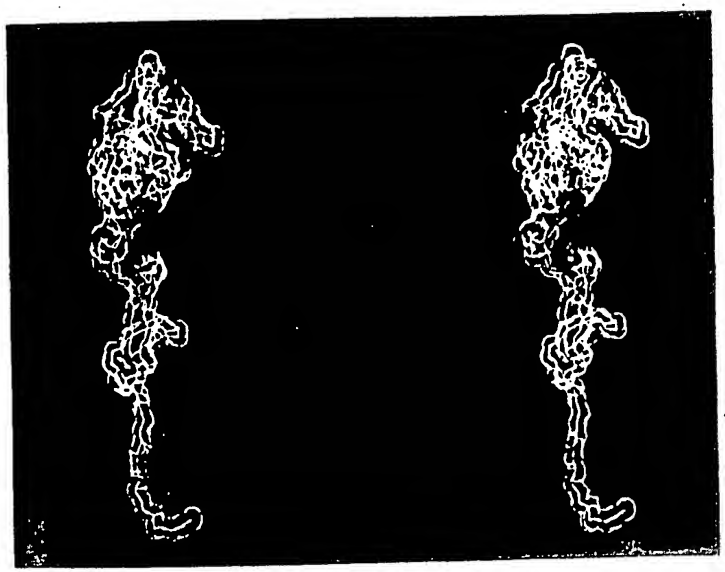

FIG. 1B.

FIG. 1. Sites on the surface of the hemagglutinin molecule to which antibodies bind (A) during infection with the whole virus, (B) using antipeptide antibodies. The α-carbon tracing of HA1 is represented by green, blue is the α-carbon tracing of HA2, and the solvent accessible surface of the antigen binding sites is expressed by purple dots.

myeloblastosis virus contains as part of its genetic information an inserted cellular sequence which is responsible for acute myeloblastic leukemia in chickens, but the protein encoded by the oncogene had not been found. They made antisera to three small synthetic peptides out of the putative 265 amino acids predicted from the long open reading frame of the virus. The use of multiple peptides from one protein is of significance for two reasons. First, since these antisera are site-specific, antisera to only one peptide might miss intermediates in a processing cascade when the part of the protein to which the antibody is directed is removed. Second, if antisera to more than one region of a protein detect the same products in cells one can be certain of the immunological specificity of the reaction, thus avoiding some of the usual problems with immunological reagents. Furthermore, if the immunological reactivity of the individual antisera is inhibited by the homologous but not heterologous peptides any possibility for spurious binding is eliminated. The three antipeptide antisera of Baluda and his colleagues behaved in concert and detected a 48,000-dalton protein from leukemic myeloblasts. Importantly the same three antisera precipitated a 110,000-dalton protein from normal hematopoietic tissue but not leukemic myeloblasts. These studies allowed the authors to conclude that $p48^{Amv}$ is the oncogenic relative of a differentiation specific normal cellular homolog ($p110^{Proto\text{-}Amv}$). These studies also raise the specter of the nature and function of the "nononcogenic" part of the 110,000-dalton molecule which might be imagined to play a role in myeloblastoid differentiation.

In another series of experiments, distinguished because they were the first to relate an oncogene to a normal protein of known function, Aaronson and his colleagues (Robbins *et al.*, 1983; Devare *et al.*, 1983) and Niman (1983) used antipeptide antibodies to demonstrate the suspected relationship between the oncogene of the simian sarcoma virus to the platelet derived growth factor. Again, one was able to gain confidence in the results since several different peptides from the amino- and carboxyl-termini (Robbins *et al.*, 1983; Devare *et al.*, 1983) and an internal fragment (Niman, 1983) were used to generate the antisera.

Antipeptide antibodies have been used extensively to investigate the proteins of the DNA containing transforming viruses. A number of different antisera to the SV40 and polyoma T antigens have been made (Walter *et al.*, 1980; Green *et al.*, 1983a; Feldman and Nevins, 1983; Luka *et al.*, 1983; Lucher *et al.*, 1983; Yee *et al.*, 1983). Additionally in the SV40 system, antipeptide antibodies were used to locate a previously uncharacterized "agno-protein" protein which accumulates late in a lytic infection and is encoded in the late leader region of the gene (Cosman *et al.*, 1982a). As for the SV40 and polyoma systems, structural and transformation associated proteins have also been studied for other DNA containing transforming viruses (Luka *et al.*, 1983; Green *et al.*, 1983b; Lucher *et al.*, 1983).

Sutcliffe, Milner, Bloom, and their colleagues have embarked on an interesting adventure which exemplifies the use of antipeptide antibodies to find the products of newly discovered genes and probably portends studies in other systems (Sutcliffe *et al.*, 1983). These investigators are interested in finding new proteins specific to certain areas of the brain. The strategy they have adopted is to clone DNA copies of brain messenger RNA and then by a process of hybridization to RNA from different tissues and different regions of the brain select clones specific for certain areas of the brain. When an interesting gene is found, it is sequenced and antibodies are prepared to several regions of the predicted protein sequence. In their first study, these investigators found two new proteins of interest. One was found in the cytoplasma and dendrites of large neurons distributed throughout the brain while the other marked a novel series of neuronal pathways and may be a precursor for a new neurotransmitter used by the pathways. The findings are probably just the beginning and one can expect major advances from this approach.

VII. Sets of Antibodies and Antibodies to Protein Domains

The special feature of all the antibodies under consideration is the fact that their specificity is predetermined and in this sense they are region or site-specific.

In some studies (i.e., where one simply wants to detect a protein) the site at which the antibodies bind is not too important, but in others knowing where they bind is the essence of the study. Again, much power is gained when the antibodies are used as sets. As mentioned above, the influenza virus hemagglutinin is a trimeric structure composed of three molecules each of HA1 and HA2. These proteins are synthesized from a single precursor which is then cleaved to form the two smaller molecules. This cleavage is a prerequisite for formation of infectious virus and part of the process by which the fusion function of the virus is activated. The process of cleavage, of course, generates new C- and N-termini of proteins, which are thought to be involved in fusion. To study these neo-termini we prepared a set of antibodies according to the strategy shown in Fig. 2. These antibodies are now being used to study the process of viral fusion with host cell mem-

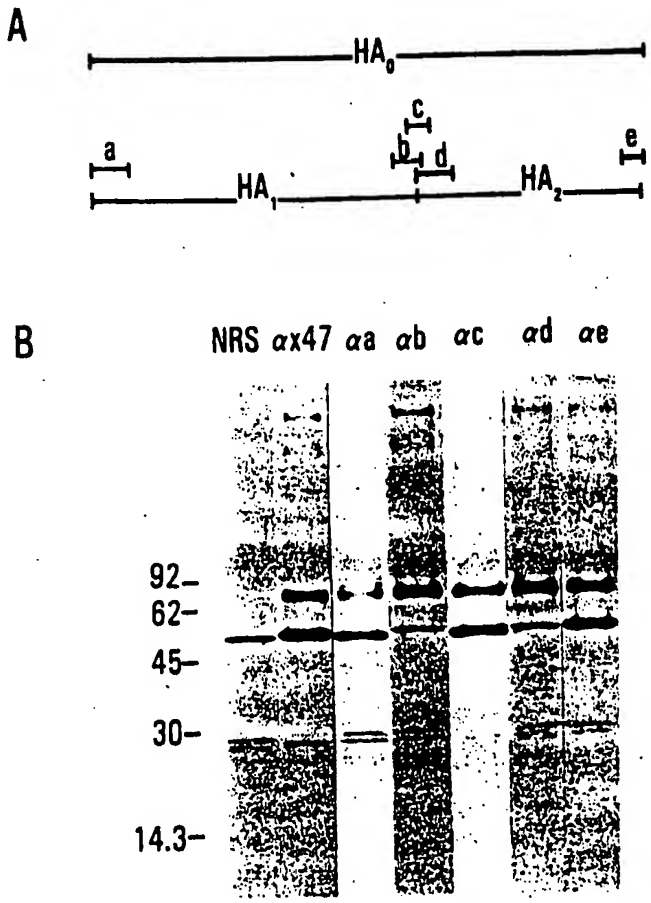

FIG. 2. Illustration of the power of sets of peptide antisera used to track distinct regions of a protein simultaneously. (A) The influenza hemagglutinin precursor (HA0) and the eventual products (HA1 and HA2). The synthetic peptides a to e, located within the HA0 sequence as shown (NH_2- and COOH-terminal, flanking and spanning the HA1-HA2 junction), were coupled to a carrier and used to immunize rabbits as described (5–7). (B) The resulting antisera (αa, αb, αc, αd, αe) and a positive control serum (antibody to X:47 influenza, αX:47) and a negative control serum (normal rabbit serum, *NRS*) were used to precipitate extracts of [^{35}S]methione-labeled influenza X:47 virus-infected cells. The five antisera to the peptides and αX:47 precipitated the HA0 molecule (this precursor is not appreciably cleaved during infection of cell lines), whereas the other precipitated proteins were nonspecific (sticky) since they also appeared in the normal control lane.

branes, but for our present purposes they serve to illustrate the generation of a set of site-specific antibodies to approach a biological problem. Hui and his colleagues also wished to make antibody to the new termini of proteins in the fibrinogen fibrin system in order to generate an antibody which would detect blood clots by reacting with fibrin but not fibrinogen (Hui *et al.*, 1983). They synthesized two peptides representative of the newly exposed amino-termini of the fibrin α- and β-chains resulting from cleavage by thrombin. They were able, by this approach, to generate fibrin-specific antibodies, a reagent which heretofore had not been available. Similarly, Sue and Sytkowski (1983) were able to generate antibodies to NH_2-terminal regions of erythropoietin.

Schneider and his colleagues (1983) have carried out elegant studies designed to demonstrate the external orientation of the low-density lipoprotein (LDL) receptor in fibroblasts. The receptor for the LDL is the major plasma protein involved in the transport of cholesterol into cells. After binding to its receptor, LDL enters into cells in coated pits via the process of receptor-mediated endocytosis (Goldstein *et al.*, 1979; Pastan and Willingham, 1981; Pearse and Bretscher, 1981; Brown *et al.*, 1983; Drickamer, 1981; Drickamer *et al.*, 1984; Beisiegel *et al.*, 1981; Anderson *et al.*, 1982). Because of its signal role in cholesterol metabolism it is critical to learn more about LDL receptors, particularly their orientation on cells. Schneider *et al.* (1983) made antibodies to a chemically synthesized peptide representative of the first 16 amino acids of the receptor. These antibodies reacted with the LDL receptor in intact, unfixed, skin fibroblasts thereby demonstrating that the NH_2-terminus of the molecule is exposed on the external surface of the plasma membrane. One of the medically important as well as technically advantageous aspects of the LDL system is that fibroblasts from subjects with homozygous familial hypercholesterolemia lack the receptor. The antipeptide antisera did not stain these receptor-negative cells, thus conclusively demonstrating the specificity of the reaction.

Maloy and colleagues (1984) were interested in a Class I H-2 molecule which is secreted rather than membrane bound (Cosman *et al.*, 1982b; Kress *et al.*, 1983). The structure was predicted from a cDNA sequence which suggested the presence of a molecule which was homologous to other Class I proteins except for the C-terminal region which had multiple base substitutions and a 13-base pair deletion. These genetic changes altered the translational reading frame resulting in a prematurely terminated protein which lacked the cytoplasmic segment and had sufficient substitutions of hydrophilic amino acids

substitutes in the transmembrane domain to abrogate its membrane spanning function. These investigators made antibodies to a region near the C-terminus of the predicted protein and have detected a new and intriguing Class I molecule (Maloy *et al.*, 1984). The sera detected a molecule of 40,000 daltons associated with β_2-microglobulin in the sera of six different strains of mice representing five distinct H-2 haplotypes. In addition the protein was concentrated in the liver which is presumably the source of its synthesis. This study represents a combination of approaches in which a new gene product is searched out by making antibodies directed to a unique site predicted by the nucleic acid sequence. In this case the frame-shift in the gene assures a unique antigenic determinant and allows one to get around the usual argument that Class I molecules in the serum are simply sloughed off cell surfaces or from degraded cells. Since gene cloning of the MHC has revealed 30–36 Class I genes (Hood *et al.*, 1983), the success of Maloy and colleagues can be expected to signal many similar studies to prepare antibodies to novel MHC gene products.

Semler *et al.* (1982), Baron and Baltimore (1982), and Morrow and Dasgupta (1983) prepared antibodies against synthetic peptides corresponding to the genome-linked protein of poliovirus (VPg). The antibodies reacted with virus-specific proteins and suggested that a membrane bound form of the viral protein, P3-9, donated VPg to viral RNA (Semler *et al.*, 1982).

Johnson and Elder (1983) prepared an antibody to a peptide predicted from the nucleic acid sequence of a replication competent, recombinant murine leukemia virus. Since the peptide was specific to a nucleic acid region acquired during the process of recombination, the antibody probe was specific for one particular recombinant envelope protein (gP70). Interestingly, this protein was found in murine thymocytes but not splenic T cells or bone marrow cells, thus giving further credence to the idea that the thymus plays a specific role in generating recombinant leukemogenic retroviruses.

VIII. Structure–Function Studies

Since one can make site-specific antibodies to virtually any region of a protein, the possibility has been opened for the fine structure mapping of regions of proteins involved in biological or enzymatic functions. This approach is exemplified by the thorough studies on the transforming proteins of Rous sarcoma virus ($pp60^{src}$). Gentry *et al.* (1983) used antibodies to positions 498–512 and 521–526 of pp60 to study the kinase site of the molecule. Immunoprecipitates prepared

using anti-498–512 failed to phosphorylate exogenously added substrates, whereas precipitates prepared with antibody to position 521–526, a region only six amino acids away, retained the enzymatic activity. In a somewhat reciprocal experiment, Tamura *et al.* (1983) showed that in the immune kinase reaction, antibodies to peptides 103–108 and 155–160 are mostly unphosphorylated, whereas antibodies to 315–321, 409–415, and 500–506 are phosphorylated to an extent similar to conventional polyclonal sera. Thus, both of these studies suggest that binding around amino acid 500 perturbs the function of the molecule, a finding consistent with the hypothesis that this region of the molecule is part of the active site. Similarly, other antipeptide antibodies have been used to inhibit the functions of proteins. Antisera to the feline sarcoma virus (*fes*) gene sequence (Sen *et al.*, 1982) and the middle T sequence of polyoma virus inhibit protein kinase activity (Schaffhausen *et al.*, 1982). Antisera to several regions in the NH_2-terminal half of the Moloney leukemia virus polyprotein inhibit reverse transcriptase enzymatic activity whereas antibodies to peptides from the COOH-terminal portions inhibit the virus-associated endonuclease activity (Sutcliffe *et al.*, 1983). Baron and Baltimore (1982) used antipeptide antibodies to p63, a core protein of poliovirus predicted from the replicase gene, to inhibit the replicase and polyuridylic acid polymerase activities indicating that both activities reside in p63.

IX. Antibodies to Proteins Encoded by Alternative Reading Frames

A variation on the idea of using antipeptide antibodies to find the products of new genes is their use in the assignment of reading frames and the detection of the products of overlapping genes. The most comprehensive study of this nature has been carried out by Mariothini and colleagues (1983) for the human mitochondria genome. When the sequence of the mammalian mitochondrial DNAs was completed, there was the discovery of eight reading frames, each over 200 nucleotides long, which did not encode any of the known translation products (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981; Grosskopf and Feldman, 1981). Mariothini *et al.* (1983) were interested in the protein product of an unidentified reading frame (URF A6L) which was 207 nucleotides long and overlapped in an alternative frame the ATPase genes by 46 nucleotides. Antibodies against two peptides from the putative amino- and carboxy-terminus of URF A6L precipitated a protein of 4500 daltons from HeLa cell mitochondrial translation products.

The other use of antibodies to alternative reading frames is to study frameshift mutations. Such antibodies can be thought of as anti—"wrong" reading frame antibodies. S. Sen (personal communication) used the nucleotide sequence of several transforming viruses to predict the protein sequence which would correspond to +1 and +2 frameshifts. Sequences were selected far enough to the 3'-end of the genome so that termination codons did not occur. These antibodies were indeed capable of detecting the frameshifts and the studies so far carried out indicate that a surprisingly high rate of frameshifting occurs. These approaches should now be extended into a variety of systems including human mutations. Aside from their uses for theoretical and medical studies, antibodies to alternative reading frames should prove useful in following the fate of transfected genes altered by site-specific mutagenesis. By using sets of antibodies one could compare the fate of wild-type and mutant proteins in the same cells.

X. Exon Usage

The genes of higher cells are a conglomerate of coding regions (exons) with interspersed noncoding regions (introns). By a process of RNA splicing, messenger RNA is assembled from larger transcripts. This lack of a direct one-to-one relationship between the DNA sequence and the final gene product sometimes makes it difficult to determine which regions of DNA encode the protein in question. Also, as for the immunoglobulin genes, which encode the secreted and membrane-bound forms of the molecule, different exons may be used at different times. Antibodies of predetermined specificity are ideally suited to follow exon usage during gene expression. Shinnick and Blattner (personal communication) followed exon usage in the IgD system by using antibodies to peptides representative of exons specific for the secreted or membrane-bound form of the molecule.

The exon usage in the adenovirus-2 E1A transcription unit has also been studied (Feldman and Nevins, 1983; Green *et al.*, 1983b). The E1A region encodes functions which both regulate the expression of other genes and play a role in cellular transformation (Berk *et al.*, 1979; Jones and Shenk, 1979a,b; Graham *et al.*, 1978). The large E1A transcript is processed into two overlapping messenger RNA molecules (12 S and 13 S) which differ by 138 nucleotides. The two messenger RNAs are in the same reading frame and translation is initiated at the common first AUG. Thus, it has been assumed that the two proteins encoded by these messages have a common N- and C-terminus and differ only by the 46 amino acids encoded by the 138 nucleotides unique to the larger message. Feldman and Nevins (1983) made an antibody to a peptide corresponding to the most hydrophilic residues of the putative 46 amino acids unique to the large protein. Indeed, this antibody reacted only with the larger of the two proteins, thus providing a reagent which should be useful in sorting out the role of E1A encoded proteins in transformation and control of transcription.

XI. The Chemistry of Virus Neutralization

Although immunological prevention of viral disease is one of the most time honored processes in medicine, the chemistry of virus neutralization is hardly understood. The basic problem has been that not all antibodies that bind to viruses neutralize them and thus it has been difficult to use populations of antibodies to sort out the problem. For example, something as seemingly simple as the serology of viral type specificity has not been easy to comprehend at the molecular level. The advent of monoclonal antibodies offered some approaches to the problem. The usual way in which monoclonal antibodies are used to understand virus neutralization is to use an approach which can be referred to as antibody escape experiments. Basically, these experiments involve growing virus in the presence of a neutralizing monoclonal antibody to select for variants which escape neutralization. The relevant proteins of the variants and wild-type strains are then sequenced (usually by nucleic acid sequencing) and the observed changes are then said to be part of the antigen binding site for the antibody. This approach has been used successfully for viruses like polio and influenza and continues to yield interesting information.

The alternative approach, of course, is to use antipeptide antibodies. Since the site on the virus or viral proteins to which these antibodies bind is known, the chemistry of viral subtype specificity and neutralization can be learned directly.

One of the first systems in which a thorough study was carried out was for the hepatitis B virus. Since this virus does not replicate *in vitro* alternative approaches were not available. The important protein target of neutralizing antibody in this system is the product of the *S* gene. The *S* gene product [hepatitis B surface antigen (HBsAg)] is a single polypeptide, of 226 amino acids, the sequence of which has been determined from the nucleotide sequence of the *S* gene (Tiollais *et al.*, 1981; Valenzuela *et al.*, 1979; Galibert *et al.*, 1979; Pasek *et al.*, 1979). The HBsAg protein consists of a group-specific (a) and two sites of subtype-specific determinants (d/y, w/r) so that four types of viruses

exist (adw, adr, ayw, and ayr) (LeBouvier, 1971; Bancroft *et al.*, 1972; Gold *et al.*, 1976; Shih *et al.*, 1978). The protein sequence of HBsAg between position 110 and 137 is the most variable and thus a candidate for a type-specific determinant (Tiollais *et al.*, 1981; Valenzuela *et al.*, 1979; Galibert *et al.*, 1979; Pasek *et al.*, 1979; Lerner *et al.*, 1981). Gerin and colleagues showed that chemically synthesized peptides corresponding to region 110–137 could duplicate the d/y specificities and induce subtype-specific antibodies even in chimpanzees which are the relevant human model of the disease (Gerin *et al.*, 1983). Initially, they synthesized peptides of 28 amino acids which differed by seven residues. By using shorter peptides the site of the y determinant was localized to one or more amino acids at positions 127, 131, and 134 of the S gene product. In other words, by using antibodies of predetermined specificity it was learned that the y/d serology is played out over no more than 3 of the 226 amino acids of the proteins. There have been a number of other experiments using synthetic immunogens to study the HBsAg. Dreesman *et al.* (1982) studied cyclic peptides between 117–137 and 122–137 and concluded that the cyclic form was beneficial in eliciting antibodies reactive with native HBsAg. Although cyclization may generate novel specificities it is obviously not necessary to generate protein reactive antibodies since linear peptides work as well (Lerner *et al.*, 1981; Gerin *et al.*, 1983). The issue of cyclization of peptides comes up often, probably because of the loop of lysozyme experiments. It is necessary to remember that a loop structure worked in lysozyme because the region in question of the native protein was, in fact, a loop. However, lacking structure evidence for a loop structure, there is no evidence that cyclization of peptides is generally helpful. The studies of Prine and colleagues stand in conflict with all the other studies of the subtype specificities of synthetic HBsAg immunogens (Prine *et al.*, 1982). They concluded that a peptide encompassing residues 138–149 contained the d determinant, whereas Bhatnagar *et al.* (1983) concluded that the same region was an essential part of the a determinant. There is nothing to say that a region of a protein cannot represent two serological determinants, but in view of the agreement between the Gerin and Dreesman studies it is probably correct to assign the d/y determinants to the C-terminal portion of peptide 110–137.

The synthetic immunogen technology has led to a thorough understanding of the chemistry of subtype specificity and virus neutralization for the foot-and-mouth disease (FMD) virus. In fact, the chemistry of virus neutralization is better understood for this than any other virus. FMD is a highly contagious affliction of cloven-hooved animals

which is caused by a single-stranded positive sense RNA genome of about 8000 nucleotides. The virus has seven distinct serotypes which can be further divided so that at least 60 subtypes of the 7 serotypes are known. Thus, this is a viral system of enormous antigenic variation which can be contrasted, for example, to another piconovirus, polio, in which there are only 3 important strains. In FMD, infection with one serotype does not confer protection against any others. Thus this viral system is, at once, an interesting model and a challenge for the synthetic immunogen technology. Basically, it combines antibodies of exquisite specificity with a viral target capable of great variability. The major target of neutralizing antibody in the FMD system is the VP1 protein which occurs in 60 copies on the virus capsid. Bittle *et al.* (1982) synthesized a number of peptides predicted from the nucleotide sequence of Kurz *et al.* (1981) and tested the antibodies to each for their ability to bind to and neutralize the virus. Whereas antibodies against all peptides bound to the virus, only those to region 141–160 neutralized the virus well. Pfaff *et al.* (1982) reasoning on the strength of a predicted surface helix of VP1 found that a hexapeptide between residues 144 and 149 elicited high titers of neutralizing antibodies. Furthermore, these authors showed that the site represented by peptide 144–149 is also a major immunogen of intact virus since it could be used to absorb out a significant amount of antibody made against the intact FMD virus. Together, these studies make a significant point about the chemistry of virus neutralization. Simply stated, there is a difference between antibody binding and neutralization and only some of the antibodies which bind to viruses are capable of neutralization. This, of course, makes great sense because if all or even a majority of antibodies to the surface of a virus were capable of neutralizing then a virus could not escape the immune system by changing one or even a few amino acids. Furthermore, as we will next see, subtype specificity is a hand-in-hand companion of a viral evasion of the immune system and this game is played out over few amino acids.

Recently an experimental system emerged which allowed an even closer look at the chemistry of subtype specificity and viral neutralization for FMD. Although, in general, antipeptide antibodies neutralized the appropriate serotypes of FMD virus, the sera against type A from the USA neutralized the Pirbright strains of A type virus poorly. The pedigree of these two A strain viruses is interesting. The Pirbright A12 virus, the parent of that used by American workers (Kleid *et al.*, 1981), had been passaged only once in BHK cells whereas the American (USA) virus had been passaged and plaque purified multiple times. When the USA virus was returned to the

Pirbright laboratories in Surrey a proper match was achieved, in that the antipeptide antibodies based on its sequence neutralized the USA virus (Rowlands *et al.*, 1983). In an attempt to understand this situation, nucleotide sequencing was carried out on the USA and Pirbright viruses (Kleid *et al.*, 1981). The nucleotide sequence of the USA virus agreed exactly (except for a Leu codon at position 212) with that previously reported (Bittle *et al.*, 1982) whereas the sequence of the Pirbright virus was ambiguous at nucleotides representing amino acids at positions 148 and 153 of the virus. These findings were of considerable interest from four points of view. First, they suggested that more than one strain of virus was present in the Pirbright stock. Second, since the virus was only one passage away from the natural host, a detailed study of the virus might offer insight into the epidemiology of the FMD strain variation. Third, the variation was largely confined to positions 148 and 153 which, it should be recalled, fall into the site (141–160) previously found to induce strain-specific neutralizing antibodies (Bittle *et al.*, 1982; Pfaff *et al.*, 1982). Fourth, a combined immunological and biochemical study of the presumed variants might offer insight into processes by which these viruses evade the immune system as well as the chemistry of virus neutralization. Accordingly, the Pirbright virus stock was plaque purified, 10 separate clones were isolated, and their nucleotide sequence was determined. Of the 10 clones three had serine at 148 and leucine at 153, five had leucine at 148 and proline at 153, and two had serine at both 148 and 153, whereas the USA virus had Phe at 148 and Pro at 153 (Brown *et al.*, 1983). Peptides corresponding to positions 141–160 for each of the viruses were chemically synthesized and antisera prepared against these. In each case, the antisera neutralized the homologous better than the heterologous viruses. Furthermore if antisera to "nonsense" peptides were prepared (i.e., leucine at 148 and 153 and phenylalanine at 148 and leucine at 153) they did not neutralize any of the viruses (Pfaff *et al.*, 1982). These results emphasize the significance of positions 148 and 153 as components of strain variation and show their critical importance in reshaping their cognate regions of VP1 during virus escape from immune attack. Obviously, some changes in these positions are more effective than others in allowing the virus to escape the immune system. For example, a leucine → proline change at 153 might effect the main chain direction and alter neutralization. Indeed, the synthetic peptides could be shown to be a good mimic of the natural serology since antibodies against the intact viruses, like the antipeptide antibodies, neutralized the homologous viruses much better than the heterologous viruses. Finally, these studies on FMD viruses have significance for our understanding of virus variation in the field. It was surprising to find that the FMD variants were in approximately equal number, especially since the variations involved only two amino acids. This should be contrasted to the situations where mutants are selected in the laboratory under host range or immunological pressures. In these situations the frequency of selection of variants is on the order of 10^4. It is not yet understood how these variants are maintained in FMD but their presence in equal numbers suggests that the changes are not disadvantageous to viral replication and are probably advantageous to evasion of the host immune system. The particulars of the FMD system aside, it is heartening to understand the chemistry of virus neutralization in such detail. The detailed X-ray structures of this and other picornoviruses such as polio (Hogle, 1984) are eagerly awaited so we can understand even more about the structure and chemistry of virus neutralization. Using antibodies of predetermined specificity we can already say what amino acids change during virus escape. When the structures are in we will be able to turn the rachet one more notch and speak of shape changes in proteins during immunological escape. At that point vaccine production will move from an empirical to a design science.

There has been success in using synthetic peptides which generate antitoxin antibodies (Audibert *et al.*, 1981; Boquet *et al.*, 1982) in the diphtheria system. Very interesting results have recently been achieved in the malaria system. Curiously, the sporozoites of the *Plasmodium knowlesi* strain and *Plasmodium falciparum* strain of malaria have surface proteins which consist of 12 repeating 12-mers and 23 repeating 11-mers, respectively (Coppel *et al.*, 1983; Godson *et al.*, 1983). In both systems antipeptide antibodies reacted with the surface of sporozoites, offering hope for a synthetic vaccine against sporozoites.

XII. Synthetic Immunogens Representing Idiotypes, Allotypes, and Growth Factors

Although the studies described above have detailed the ability to make antibodies to variable portions of proteins, we deal separately here with the variable regions of proteins which are of special interest to the immunologist. Also included in this section are studies using antipeptide antibodies to study lymphokines.

Perhaps the most telling study concerning the ability of antipeptide antibodies to achieve fine specificity is that of Alexander *et al.* on the

Thy-1 glycoproteins (Alexander *et al.*, 1984). The Thy-1 glycoprotein exists in two allotypic forms which differ from each other by a single residue (Reif and Allen, 1964).

The protein with an arginine residue at position 89 is thought to correspond to the Thy-1.1 allotype and a glutamine residue to Thy-1.2 (Williams and Gagnon, 1982). Since the two proteins differ by only a single amino acid they offered a good test system to study the ability of antipeptide antibodies to discriminate between two closely related proteins. Based on the protein sequence (103) six peptides were synthesized. Four peptides corresponded to the constant region of the molecule, whereas two spanned the region between amino acids 79 and 98 which correspond to the only known differences between the 1.1 and 1.2 alloantigenic forms of the Thy-1 molecule. Antibodies to the peptides from the conserved regions of the protein were reactive with both Thy-1.1 and 1.2, whereas antisera to peptides spanning the variable regions which were predicted to be from Thy-1.2 and 1.1 molecules showed the corresponding preference for thymus extracts of the C57BL/6J (Thy-1.2) and AKR/J (Thy-1.1) mice.

The peptides utilized in this study are of sufficient size that each could contain multiple antigenic determinants. Indeed, these peptides have 18 amino acids in common and differ with respect to only one. Nevertheless, the presence of a single distinguishing amino acid is sufficient that a significant portion of the immune response toward each peptide is specific, resulting in preferential reactivity of the antisera with their inducing peptides. Hence, the free peptides closely mimic the differential immunogenicity of the native Thy-1 alloantigens, and confirm the suggestion that amino acid 89 is involved in Thy-1 alloantigenicity.

Of the two chemically synthesized sequences, the one with Arg at position 89 elicits antibodies with a higher degree of specificity than the one with Gln. Thus, amino acid changes can either render or abolish differential immunogenicity. In the present study replacement by *Gln* by *Arg* at position 89 diminishes differential immunogenicity and the resultant peptide appears now to be composed of a collection of more equally potent antigenic determinants.

It is interesting to point out that the same phenomenon has been observed in other systems as well. In the hepatitis B surface antigen (HBsAg) system we constructed a peptide in the region of amino acids 110–137 which induced antibodies with a strict specificity for the adw subtype of HBsAg. The substitution of two amino acids within this peptide (asparagine and phenylalanine at positions 131 and 134 were

replaced by threonine and tyrosine, respectively) resulted in a peptide with a broader immunological specificity.

The findings in the Thy-1 system are of theoretical interest from several points of view. First, single amino acid changes can "control" the immunogenicity of relatively long peptides. This is, perhaps, not too surprising for the induction of antibodies which react with the free peptide, but when we realize that the antibodies also discriminate between the intact folded proteins some interesting concepts emerge. Obviously, since allotypic forms of Thy-1 exist, one already knew that the immune system could differentiate the single amino acid change in the context of the folded protein, but this could have been due solely to complex changes in conformation caused by the Gln and Arg change. The data show that the differential immunogenicity of Thy-1.1 and Thy-1.2 can be due rather to a local perturbation in structure effected by this change. An important point which emerges from this study is that even a single amino acid substitution can result in the formation of allotypic antigens where the allotype can be detected by antisera in different ways. For example, the antipeptide antibodies efficiently detect both the synthetic peptides and the isolated allotypic Thy-1 glycoproteins, whereas monoclonal anti Thy-1 allotypic antibodies are efficient in the detection of these intact alloantigens, but do not detect these synthetic peptides (H. Alexander *et al.*, personal communication). Another difference between these antipeptide antisera and monoclonal anti Thy-1 antibodies is that the latter are much more efficient in the detection of cell-surface Thy-1 alloantigens. This is not surprising since, classically, monoclonal and monospecific anti Thy-1 alloantibodies were selected for their ability to bind to the cell surface of Thy-1. There are advantages to the antipeptide antibodies for certain practical uses. It is theoretically possible to use this technique to produce antibodies to other single amino acid-defined polymorphic and mutant polypeptide sequences and to use such antibodies to detect gene sequences encoding them. For example, there is preliminary evidence that the antipeptide antisera readily detects Thy-1 antigens in a bacteriophage expression system into which a T cell-derived cDNA has been inserted, while in contrast, the monoclonal antibodies fail to do so (Rosen *et al.*, unpublished data).

Technical details aside, the important question is why should a single amino acid change similarly affect the immunogenicity of a peptide in solution and the cognate structure in the internal region of a folded protein, where its conformational flexibility is likely to be constrained by bonds with neighboring structures. One possibility is

that the peptide in solution has a favored conformation which closely resembles that of the folded protein. This is, however, inconsistent with the body of evidence which suggests that in aqueous environments, even relatively long peptides have very little ordered structure (reviewed in Goetzl and Peters, 1972). This and other theoretical aspects of the problem will be discussed below.

Altman and his colleagues prepared antipeptide antibodies reactive with human interleukin 2 (Altman *et al.*, 1984). They chemically synthesized 8 peptides, each consisting of 13–15 amino acids. Antibodies to four of the eight peptides reacted with native IL-2 as judged by biochemical and immunological criteria. Furthermore, the antisera were able to react *in situ* with IL-2 producing cells. Cells stimulated with PHA but not control cells were stained with affinity purified rabbit antibodies against the synthetic peptides corresponding to IL-2. It seems reasonable to suspect that the IL-2 story is only the tip of the iceberg as far as factors which regulate immune functions are concerned and thus one might expect antibodies of predetermined specificity to play an increasing role in unraveling the functions of these factors. This is especially true for two reasons. First, it is likely that the factors will come to light via nucleotide sequencing, and thus predicted protein sequences will be known in advance of any formal protein chemistry. Second, as for other hormone systems there are likely to be polyproteins involved and these antibodies will be useful in sorting out precursor–product relationships.

McMillan *et al.* (1983), Chen *et al.* (1984a), and Seiden *et al.* (1984) have chemically synthesized idiotypic determinants of mouse and human immunoglobulins. Working with the third hypervariable region of murine antidextran antibodies, McMillan and colleagues synthesized peptides corresponding to sequences in the M104 and J558 myeloma proteins. Rabbit antisera to these peptides reacted only with heavy chains and appropriately discriminated between the M104 and J558 proteins. Chen *et al.* (1984a) studied the monoclonal IgM rheumatoid factor (Sie) using the sequence of Andrews and Capra (1981). A peptide corresponding to amino acid residues 99–111 reacted with the IgM Sie protein but not with four other IgM rheumatoid paraproteins or with pooled human IgG. These results indicate that in this system the antipeptide antibodies are reacting in this system with a private idiotype on the heavy chain of the Sie rheumatoid factor. In a separate study Chen *et al.* (1984b) chemically synthesized peptides corresponding to the "Wa" marker on the complementarity determining region (Kunkel *et al.*, 1973, 1974) of human monoclonal IgM rheumatoid factor (RF) autoantibodies. The Wa marker is of particular

interest because it is related to light chains (Kunkel *et al.*, 1974; Andrews and Capra, 1981; Carson and Fong, 1983) and is found in 60% of monoclonal IgM-RF proteins. The antipeptide sera were specific for the light chains, the IgM-RF paraproteins, Sie, Glo, and Teh but not Lay, confirming the reactivity with the "Wa" idiotypic determinant.

The experiments of Seiden and colleagues have yielded perhaps the most surprising results concerning synthetic idiotypes. They synthesized a 16 amino acid peptide corresponding to a sequence in the JH_1 segment of the murine anti-α (1–3)-dextran immunoglobulin family. Of the 20 anti-α (1–3)-dextran antibodies so far sequenced 13 contain JH_1, 5 contain JH_2, 1 JH_3, and 1 JH_4 (Clevinger *et al.*, 1980a, 1981; Shilling *et al.*, 1980). Seiden thus expected to have an antibody which uniquely bound to JH_1 containing antidextran antibodies. The surprising result was that all 16 antidextran antibodies tested bound to the antibody whereas 14/15 antibodies with different antigenic specificities did not (the exception was HOPC-1). These authors labeled this idiotype JH-Dex. The reason for the JH-Dex specificity with the antipeptide antibodies is not yet clear, but Seiden *et al.* have suggested that it may be related to the very short D segments in the antidextran antibodies. Some support for these notions can be gleaned from computer graphics analysis of the crystal structures of solved immunoglobulin molecules (A. Olson, unpublished). Usually, the D segment amino acids would "cover" the J region, and thus the shortening of the D segment in antidextran antibodies may make more of J accessible to antiidiotypic antibodies (Seiden *et al.*, 1984). Alternatively, any change in D could alter in other ways the conformation and/or the availability of the J region so as to make union with antibody more efficient.

In toto, the four experiments discussed above indicate that the site-specific nature of antibodies made against synthetic idiotypes will allow one to work out the chemical basis of idiotypic markers. Site-specific antiidiotypes could also be used to perturb the antigen binding functions of antibodies and thus lead to a better understanding of the structural correlates of antigen–antibody union. Finally, synthetic idiotypes could be of considerable medical importance. For example, in those autoimmune diseases where the offending antibody is of restricted origin, it may be possible to use synthetic immunogens to modulate or even eliminate the clones producing the antibody. Alternatively, some antiidiotypes may block or obstruct the binding pocket so as to preclude union between the injurious antibody and its antigen. Several points illustrated in the above studies are significant as far as the potential medical applications of synthetic idiotypes are

concerned. First, antiidiotypes induced by synthetic immunogens react with native immunoglobulins. Second, synthetic idiotypes offer a degree of specificity which could not be obtained by immunizing with the intact immunoglobulins. Third, and perhaps most important, because synthetic peptides can induce antibodies to regions of a protein not ordinarily recognized (see above), the possibility exists for terminating tolerance to restricted regions of self-proteins. The finding and synthesis of a common idiotype for a human autoantibody may open up an interesting route to therapy of diseases of monoclonal origin that are not under "antigen-drive." The difficulty in dealing with these diseases under "antigen-drive" is that if you could use synthetic peptides to immunize against a clone with one idiotype, upon its disappearance the immune system may reward you with an even more harmful clone (i.e., one with a slightly different antibody of higher affinity.

XIII. The Structure of an Antigenic Determinant in a Protein

One of the most powerful aspects of the synthetic immunogen technology is that it offers a means to understand the chemistry and structure of antigenic determinants in proteins. The key phrase is antigenic determinants *in* proteins as opposed to immunogenic determinants *of* proteins.

Almost all previous studies of protein immunogenicities depend on defining antigenic determinants after immunization with the whole protein. Consequently, it is difficult to localize precisely the antibody-combining site and to define the extent and number of amino acids directly involved in antibody–antigen union. Changes of amino acids in antigenic determinants which alter antibody binding and specificity may not always be easy to interpret in the context of complex structures. For example, changes in one region of the protein may in some circumstances alter the conformation of quite distant regions.

The generation of antipeptide antibodies which have predetermined sequence specificity would seem to offer an alternative approach to the problem of understanding the general nature and structure of antigenic determinants and antibody–antigen interaction. The study of antigenic determinants in a protein defined by antipeptide antibodies differs from the study of immunogenic determinants of proteins which depend on immunization with the intact protein, in that the latter determinants are often more complex and less amenable to structural study. Wilson and colleagues used monoclonal antipeptide antibodies to study the chemical nature and structure of antigenic

determinants in the influenza virus hemagglutinin (Wilson *et al.*, 1984). This study is detailed here as a prototype for the structure of an antigenic determinant in a protein. Parts of the text which follows and Figs. 3–8 are reproduced with permission from *Cell*.

Monoclonal antibodies with predetermined sequence specificity were generated by immunizing mice with a synthetic peptide coupled to KLH. The synthetic peptide represents residues 75–110 in the HA1 chain of A/Victoria/3/75 (X:47, H3 subtype) hemagglutinin (Min Jou *et al.*, 1980) when aligned with the A/Aichi/2/68 (X:31, H3 subtype) sequence (Verhoeyen *et al.*, 1980). Twenty-one different monoclonal antibodies were raised as described in Niman *et al.* (1983). The majority of these antibodies (16 out of 21) cross-reacted strongly with X:47 influenza virus. Thus these antibodies recognized the peptide against which they were raised as well as the whole virus.

The synthetic peptides which specify the binding sites (1–4) are shown in Fig. 3. Wilson *et al.* (1984) synthesized several smaller, overlapping peptides to localize the sequences to which antibodies bind with binding titers approximately equivalent to that of the parent protein.

Eight of the 18 monoclonals reacted with peptides localized to one sequence of the parent molecule. These hybridomas bind to residues in the 36 amino acid synthetic peptide that contains HA1 residues 98–106. Three hybridomas also bound in this region but could not be localized further than to sequence 88–110.

Three antibodies could be localized to bind to sequences on the amino-terminal end of the longer peptide. These binding sites overlap and are identified as site 1, 75–86, site 2, 79–86, and site 3, 83–92.

The immunodominant region, site 4, which corresponds to sequence 98–106 is shown in Fig. 4. This antigenic determinant corresponds to no more than 25% of the total amino acids in the sequence 75–110 and in this conformation has an accessible surface area of 763 $Å^2$ (24% of the total). Site 2, residues 79–86, has an accessible surface area of 800 $Å^2$ when calculated in the conformation it has in the native protein. The dimensions of sites 2 and 4 are approximately 13 × 22 × 12 and 12 × 22 × 14 Å, respectively. These structural renderings represent an idealized situation for envisaging recognition by antibody of both the free peptide and the intact protein.

As discussed by Wilson and colleagues, analysis of the structure of the antigenic determinants of the synthetic peptide is hampered at present by uncertainty about the conformation of the free peptide in solution. However, many of these difficulties are abrogated by analyzing the structure of the peptide and the antigenic determinants in the

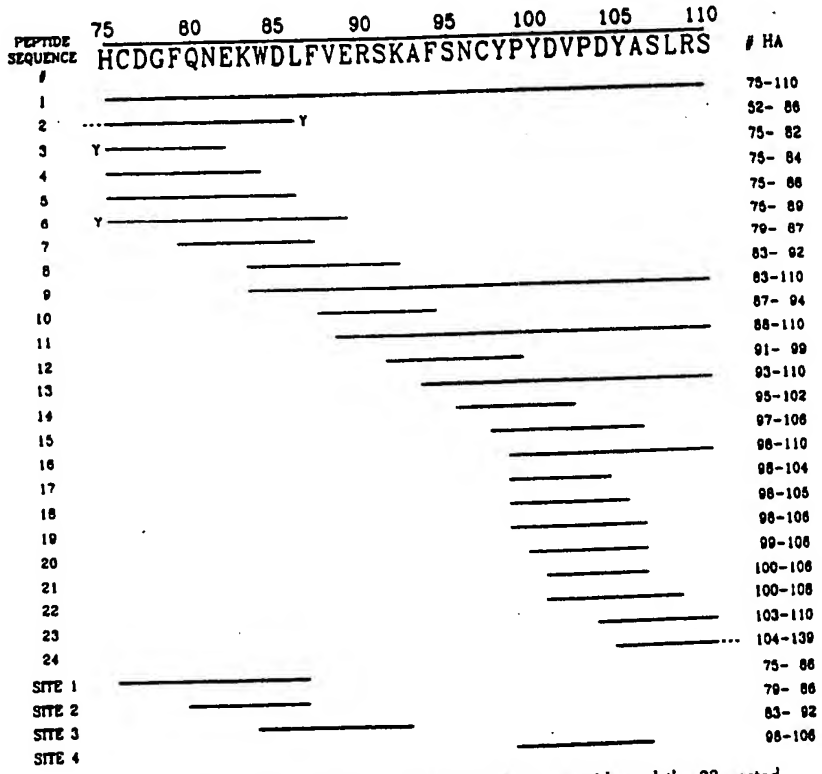

FIG. 3. The amino acid sequences of the HA1 75–110 peptide and the 23 nested fragment peptides used in the experiments are shown relative to each other. Peptides 2 and 24 actually extend below residue 75 or beyond residue 110, respectively. Three peptides (Nos. 2,3,6) have a tyrosine prepended or appended to the fragment, designated with a Y. Sites 1–4 correspond to the locations where the vast majority of monoclonal antibodies bind.

native protein. Since the antibodies have approximately equivalent binding titers with the free peptide and intact protein, it is reasonable to assume that the antigenic determinants are shared by both.

The location in the hemagglutinin molecule corresponding to the 36 amino acid chemically synthesized peptide is central in the globular head region of the HA1 polypeptide chain (Fig. 3, Wilson *et al.*, 1981). This peptide consists of six pieces of extended polypeptide chain connected by a series of bends. The amino- and carboxyl-ends

of the chain contain three bends and a helix. The peptide contains residues which are either on the surface (75–83, 91–96), buried in the monomer (83–91, 97–99, 108–110) or in the trimer interface (100–107) (Fig. 5). The sequence 83–91 contains the largest stretch of residues of the peptide inaccessible to ligands in its native conformation. When considered as part of the native monomeric protein, the peptide has an accessible surface area of 1293 Å^2, which corresponds to 41% of that of the free peptide. The accessible surface area of the peptide in the trimeric hemagglutinin molecule (Fig. 6) is even more limited.

On the outside of the molecule, the peptide thus presents a continuous surface (Fig. 8, blue surface), where residue 75 is close to residues 96 and 97, such that residues 96–97 and 75–83 form a continuous belt of accessible surface. The residues which are in the trimer interface (100–107, Fig. 8, white surface) are accessible only to water molecules, small ligands or molecules with small protruding surfaces.

Chemical identification of the residues involved in recognition of the synthetic peptide by the monoclonal antibodies together with analysis of the location and conformation of the peptide in the native structure were combined to describe the structure of the antigenic determinants in the protein. The peptide 98–106 is detailed here as this is the sequence which appears to be immunodominant.

This sequence encompassing residues 98–106 lies in the trimer interface and has accessible surface only to small ligands for residues Tyr 100, Asp 101, Pro 103, Asp 104, Tyr 105, and Ala 106 (Fig. 7). The peptide binding studies indicate that the binding can probably be localized to residues 100–106. These residues form a fairly flat, slightly convex surface, embedded in the rest of the protein, with approximate dimensions 16 × 15 × 7 Å. This peptide contains two prolines, two aspartates, and two tyrosines and may be more conformationally restricted due to the more limited torsional angles of proline residues. Unless the antibody hypervariable loops can protrude into the cavity in the trimer interface, the structure and binding data indicate that the sequence would be more accessible to an antibody binding to the monomer.

Wilson *et al.* (1984) suggested that antibody binding occurs to a structure in which the hemagglutinin monomeric heads are exposed and which represents a conformation different from the native hemagglutinin trimer.

Thus, an important conclusion from these and previous studies is the suggestion that antipeptide antibodies can have access to structures other than the native conformation, and the protein displays

conformational mobility at least in local regions. Indeed, conformational changes in the hemagglutinin molecule have been identified previously. For example, at low pH (pH 5.0–pH 5.5, Skehel *et al.*, 1982) the molecule becomes accessible to cleavage by trypsin to obtain monomer head structures with altered antigenic activity (Daniels *et al.*, 1983). In addition, unless amino acid substitutions affect distant regions in the molecule, one of the sites in the hemagglutinin to which antibodies against the intact molecule bind (site D, Wiley *et al.*, 1981) would also require a conformational change in the hemagglutinin. Site D is buried in the trimer interface and, in terms of its inaccessibility, is similar to site 4 in the trimer and site 3 in the monomer. The complete determination of the structure of these and other antigenic determinants awaits the determination not only of the peptide conformation but also of the conformation of the peptide and intact protein when complexed with an antibody. Such studies are in progress in Wilson's as well as other laboratories.

FIG. 4.

FIG. 4. The solvent-accessible surface of the peptide 75–110 is shown on a raster display unit. The grey transparent surface reveals the ball-and-stick representation of the atoms of residues 98 to 106 of the peptide. The conformation of the peptide is that it would have in the native hemagglutinin. The carbon, nitrogen, and oxygen atoms are colored green, blue, and red, respectively. The nontransparent yellow surface covers the rest of the 75–110 region.

FIG. 5. The location of the peptide sequence 75–110 is shown on the schematic diagram of the X-31 influenza hemagglutinin monomer. The HA1 chain is shown in blue, the HA2 chain is shown in cyan. The peptide 75–110 is shown in yellow with the subsequences 79–86 and 98–106 highlighted in orange. The numbers of the α-carbons are also shown. The figure, as with figures 3–8, was generated using the coordinates provided by Wilson, Skehel, and Wiley (unpublished).

FIG. 6. The hemagglutinin trimer is shown looking down the 3-fold axis of symmetry from the end furthest from the membrane with the solvent accessible surface of the peptide 75–110 represented by dots. HA1 is colored blue, HA2 cyan. The surface of subsequence 98–106 is shown in red while the rest of the peptide's surface is in yellow.

FIG. 7. The solvent-accessible surface around the subsequence 98–106 in the HA1 chain of hemagglutinin is shown in a closeup view on a raster display terminal. The grey transparent surface reveals the ball-and-stick representation of the atoms of residues 98 to 106 of the peptide. The carbon, nitrogen, and oxygen atoms are colored green, blue, and red, respectively. The nontransparent blue surface covers the rest of the 75–110 region, the yellow surface covers the HA1 chain around the 75–110 sequence and the cyan surface covers the nearby HA2 chain.

FIG. 8. The solvent-accessible surface of the hemagglutinin dimer highlighting the position of peptide 75–110 and its subsequence 98–106 is shown on a raster display unit. The left monomer shows HA1 in red, HA2 in grey; the right monomer shows HA1 in yellow and HA2 in cyan. On both monomers, residues 98–106 are colored white and the rest of the residues of peptide 75–110 are colored blue.

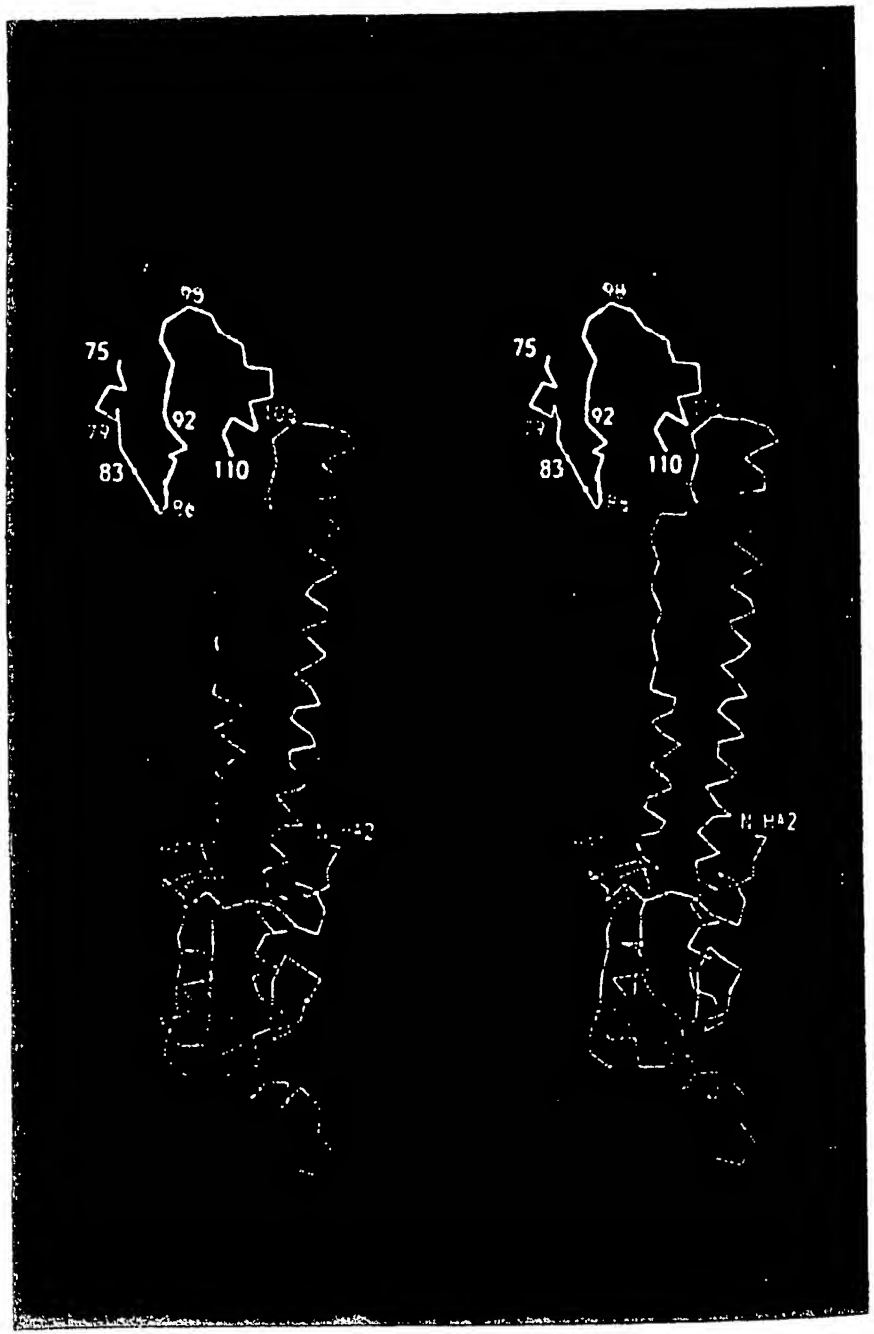

FIG. 5.

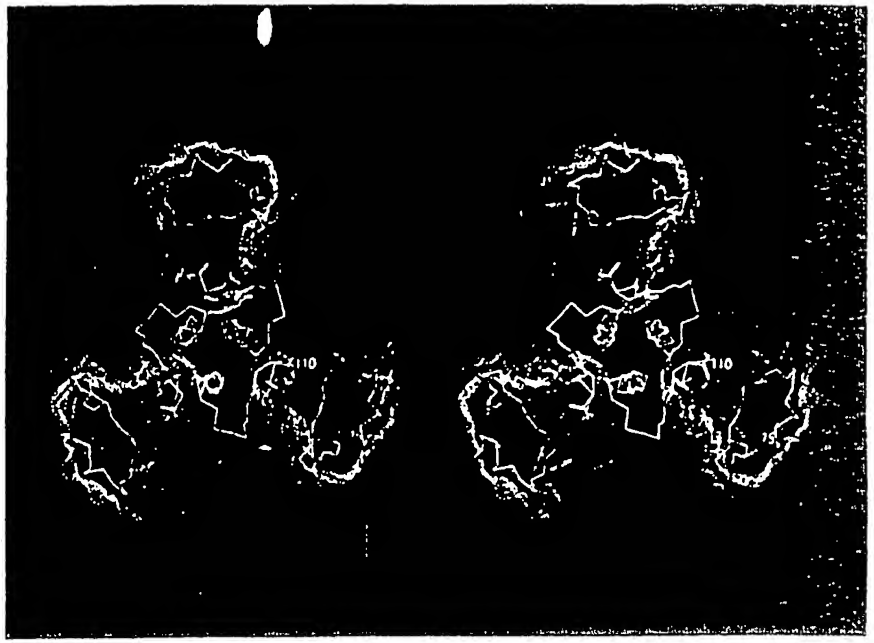

FIG. 6.

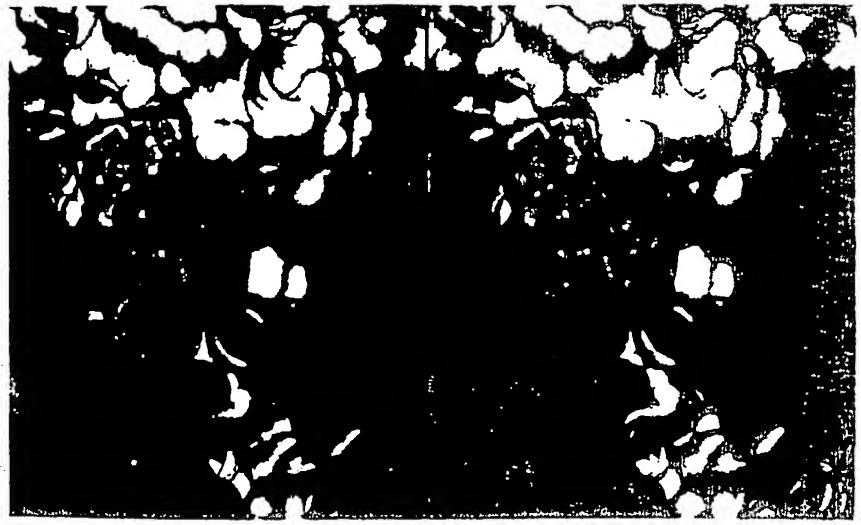

FIG. 7.

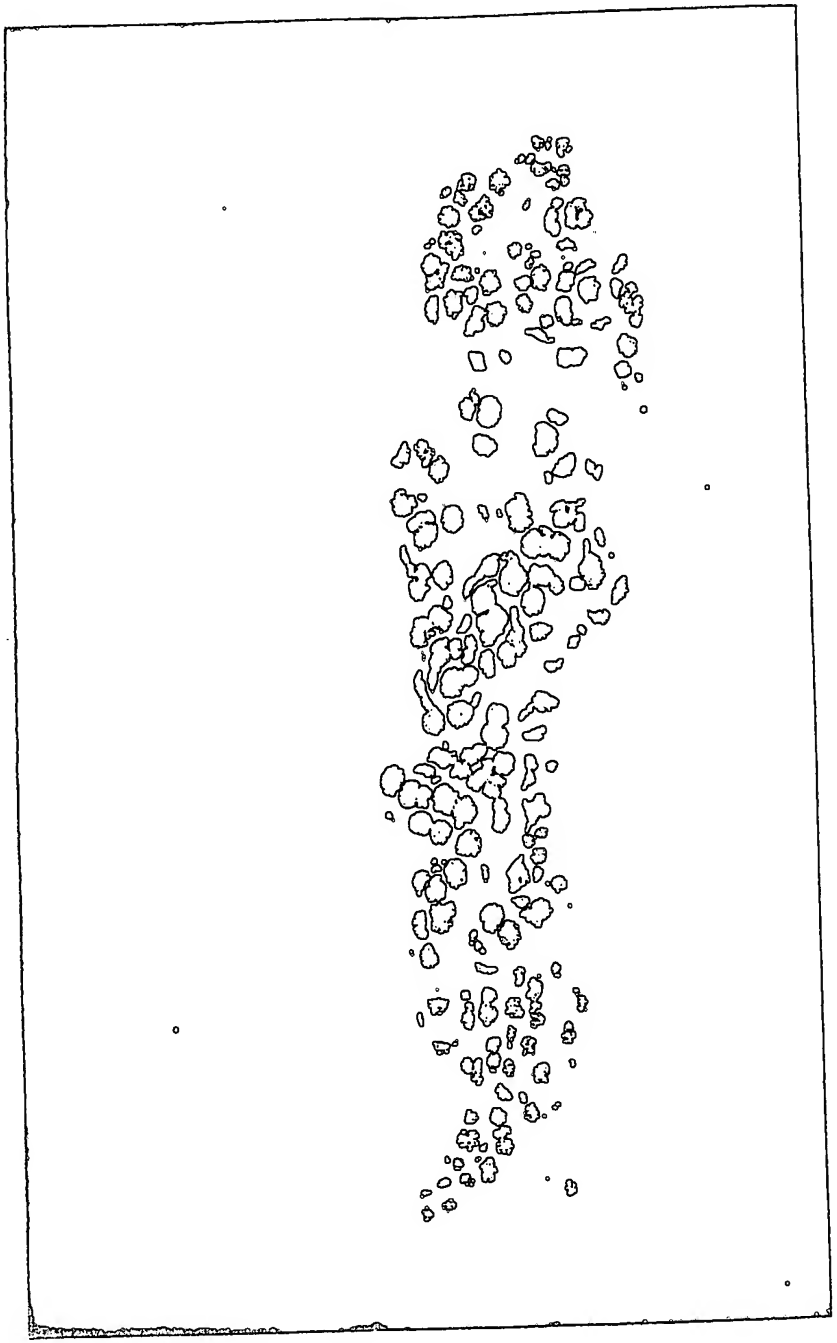

FIG. 8.

It is interesting to consider the nature of antigenic determinants in terms of what is known about antigen-combining sites of immunoglobulin molecules. The structural determination of multiple myeloma proteins (reviewed by Amzel and Poljak, 1979) of intact IgG molecules or fragments such as Fab have shown the hypervariable regions to be located around a cleft at the distal ends of the molecule. Binding studies with multiple myeloma proteins have shown that small ligands such as vitamin K (Amzel *et al.*, 1974) or phosphorylcholine (Segal *et al.*, 1974) bind to antibodies in this region but occupy only a small portion of the potential antigen-binding site. Characterization of the binding sites of hybridoma antibodies specific for (1–6) linked dextran showed that an antigen of about 5–7 hexoses was complementary to the binding site of IgA and IgG antibodies (Sharon *et al.*, 1982). Wilson *et al.* calculated the accessible surface of the pocket formed by the hypervariable residues of Feb Neu (Amzel *et al.*, 1974) and estimated the potential binding site to be 44 × 35 × 34 Å in dimension with an area of 2400 Å.^{2} The antigenic determinants of the free peptide reported by them have dimensions in site 1 of 18 × 23 × 14 Å, site 2 of 13 × 22 × 12 Å, site 3 of 29 × 13 × 11 Å, and site 4 of 12 × 23 × 14 Å and with accessible surface areas in the protein of 850–1160 Å^2. Thus, these determinants would occupy a sizable portion of the antigen-binding site of the antibody molecule.

XIV. Technical Aspects: The Only Rule Is That There Is No Rule

The question often arises as to which peptides should be made to generate protein reactive antibodies. It is going to be difficult to come up with generalities since what is learned about one peptide will not necessarily relate to a different peptide or even a similar peptide in the context of a different protein. My own view is that with certain exceptions (see below) th main issue which should guide selection of the peptide is the precision with which a given antibody can answer the experimental question at hand. As the technology has moved from simply a way to access a protein predicted by a nucleic acid sequence to a site-specific methodology, the experimenter often has a favored site in the protein against which he/she wishes antibody to be generated. There are now enough examples where this has worked to suggest that one should simply work exactly with the regions of interest. So even if an area adjacent to the primary candidate seems more attractive (i.e., more hydrophilic) one should (within reason) serve the needs of the experiment rather than attempt to guess about the limited to nonexistent "rules" governing immunogenicity. Having

said that, we can consider a few hints. The incorporation of hydrophilic amino acids in the peptides is useful from two points of view. First, peptides containing such amino acids are likely to be soluble and thus easier to work with. Second, as mentioned above, even if one could generate antibodies to very hydrophobic peptides, the cognate sequence in the protein is likely to be buried and thus inaccessible to antibody. Another good strategy is to design the synthesis so that one obtains two immunogens for each study. Unless there are other constraints, we generally make peptides of 15 amino acids in length. During synthesis we take one-half the yield at 8 amino acids and then continue until we reach 15—thus two immunogens are generated in one synthesis. Since synthesis proceeds in the C-terminus → N-terminus direction, we put a cysteine on the C-terminus so both the 8-mer (9-mer if the artificial cysteine is counted) and the 15-mer can be coupled using the same C-terminal cysteine. Sometimes it is necessary to use very short peptides. For example, in the work on synthetic idiotypes, the peptide immunogens had to be sufficiently short so as not to extend into the constant regions of the molecule. In cases like this it is often good to extend the peptide by using spacer amino acids. In the idiotype work (McMillan *et al.*, 1983), spacers containing proline and glutamic acid residues were found to be suitable.

Many different coupling methods and carrier proteins have been used in the generation of antipeptide antibodies. We usually couple the synthetic peptide to KLH through a cysteine residue but there is no evidence that this is superior to a variety of other procedures. If one is going to use the antibodies to react with the products of cultured cells it is probably best to avoid using BSA as a carrier, as the antialbumin antibodies can give spurious results since so many ligands and proteins bind to the albumin contained in the bovine serum in the culture of cells.

XV. Theoretical Aspects

As more and more data concerning antipeptide antibodies have been gathered, a theoretical construct has begun to emerge. At first, there were two rather simplistic notions to explain the results. The first idea could be called the side chain constellation theory. Basically, the notion was that antipeptide antibodies "saw" a collection of side chains as if they were a set of haptenes on a protein backbone. It was as if antibodies read the sequence of a protein. Given that any type of protein–protein binding, whether it involves antibodies or enzymes, is an interaction between specific shapes, this was never a strong idea.

However, to test this notion, we synthesized the C-terminal 20 amino acid of the influenza virus HA1 chain in four different ways: from L-amino acids in an N → C and C → N direction and from D-amino acids in the N → C and C → N direction (Houghten and Lerner, unpublished). The D isomer polymer in the C → N direction (retro-D) is particularly interesting in that, except for the ends, the relative positions of the side chains approximate the L isomer in the N → C direction but the positions of the carbonyl group and the amide bonds are reversed. In other words, we have a polymer with the same sequence but the wrong shape. Antibodies were made to each of the four peptides and tested for reactivity with the peptides as well as the folded HA1 protein. All four peptides raised antipeptide antibodies, but each reacted only against the peptide against which they were generated: only the antibody against the L, N → C peptide reacted with the HA1. The absolute lack of cross-reactivity between any of these four antipeptide antibodies speaks strongly against notions that depend on antipeptide antibodies reacting with a constellation of side chains.

The second theory evoked a stochastic model. Here, the idea was that after peptide immunization, antibodies were made against the multiple peptide conformations but only the small fraction of antibodies against the conformation shared between the peptide and protein was reactive with the folded protein. Thus, the success of the technology was postulated to be more a testimony to the sensitivity of immunological assays which could detect a small percentage of proper antibodies than to something more fundamentally interesting. However, peptides in solution have thousands to hundreds of thousands of conformations and as more and more success for different peptides was achieved, the stochastic idea became less tenable. In other words, the scientific community was doing the statistics and the collective answer was against the stochastic model. To test the stochastic model in a formal way, Niman *et al.* (1983) used monoclonal antipeptide antibodies as a way of estimating the frequency with which small peptides induce antibodies that react with folded proteins. They made monoclonal antibodies to six chemically synthesized peptides from three proteins. The frequency with which the peptides induced protein-reactive antibodies was at least four orders of magnitude greater than expected from previous experimental work and vastly different from what would be predicted by calculating the possible number of peptide conformers in solution. These results suggested that sufficient structural information is contained in peptides as small as 13 amino acid residues to induce protein reactive antibodies at a high frequency. The key point was that the frequencies observed were inconsistent with any stochastic model.

If the stochastic model is also not correct, then, with some caveats which we will discuss below, we must begin to think in terms of antibodies reacting with conformations in proteins which are different from that of the native. These notions open up the exciting possibility of a merger between the protein dynamics and the antibody problems. In the first instance one can imagine that a given site in a protein is mobile and that as it passes through a conformation which the antibody recognizes, union takes place. A second possibility is that the antibody–antigen union, itself, induces a shape change. The end result of both models is the same in that antibody is bound to a protein with the site of union in a different conformation from native. But the two models are vastly different insofar as their implications for protein structure are concerned. The first model views proteins as highly dynamic structures, whereas the latter makes no such assumptions and assumes that antibody–antigen union has sufficient energy to distort the target structure. The difficulty with the latter model is that it is somewhat circular in that antibodies can only distort that which they can recognize, and distant conformations would not be efficiently recognized. Thus, all facts considered, it seems likely that for some percentage of the time, local disorder occurs on short segments of the protein, allowing reaction with antipeptide antibodies. This local disorder model suggests that peptides that elicit antibodies recognizing intact proteins are located in areas of relatively great conformational mobility in the intact protein.

Whichever model one favors, it is important to note that peptide immunogens do not have the same possibility for induced fit that occurs in systems with a limited number of receptors (i.e., peptide hormones) because the immune system is a system of diversity, and unless other factors pertain (see below), "fixing" the correct conformation would seem to have no better probability than fixing the incorrect one.

As mentioned above, there are a number of caveats which should be considered. It can be argued that the immune system may recognize preferentially the conformation of a peptide shared by native proteins. Although this seems to be highly unlikely at first consideration, most proteins coevolved with the immune system, and "preexposure" is, in fact, possible, particularly in the case of the viral proteins. In other words, existing proteins may have played a role in shaping the immunological repertoire. The argument against this view is, of course, that one can make antibody against virtually any nonprotein chemical. But this could simply be a "side show" due to the cross reactivity between proteins and chemical antigens so that any antichemical antibody is in

reality an antiprotein antibody where the binding constant for the protein would be much higher if the correct determinant could be found.

Factors involved in the presentation of the peptide antigens may also restrict the range of conformations available to the peptides, making the native conformer far more common than predicted for the free peptide in aqueous solution. For example, the membranes of presenting cells may provide an environment that limits peptide conformation. The peptides are usually coupled to carrier proteins, and interaction of the peptide with the surface of the carrier could be expected to greatly restrict the conformations accessible to the peptide. Peptides could have a more stable native-like structure than has been suggested from previous theoretical and experimental studies with model peptides. For example, peptides may contain particularly stable local structures like those shown by others (Brown and Klee, 1976; Bierzynski and Baldwin, 1982; Kim and Baldwin, 1982; Bierzynski *et al.*, 1982). Stable local structures could serve as initiation sites in the folding of nascent or denatured proteins. If true, such a model would have important implications for the general problem of protein folding (Tanford, 1968, 1970; Anfinsen and Scheraga, 1975; Nemethy and Scheraga, 1977; Creighton, 1978; Privalou, 1979; Jaenicke, 1980; Ptitsyn and Finkelstein, 1980; Thomas and Schechtor, 1980; Wetlaufer, 1981; Richardson, 1981; Rossman and Argus, 1981; Kim and Baldwin, 1982; Levinthal, 1968).

XVI. The Repertoire Should Be Tapped Further: Concept of Immunological Catalysis

The advent of antibodies of predetermined specificity will condition us to think in terms of binding to specific sites in proteins rather than to just proteins. One wonders if the next step can be taken and antibodies can be produced which bind to the same structures in proteins as do enzymes. Since the effectiveness of enzymes depends upon the stabilization of minor equilibrium states we might expect antibodies recognizing these same states to carry out catalytic functions. This is a wonderful possibility since one could fish in the immunological repertoire for any kind of enzyme so long as the substrate were sufficiently large to be immunogenic. The basic question is whether the diversity of the immune system is any match for the millions of years of molecular design which go into the evolution of an enzyme. I, for one, would bet on the diversity of the immune system. There is a real impetus to test those notions because, since we can

now make antibodies to almost any position on a protein, success would be tantamount to having site specific enzymes for proteins. Such enzymes would be analogous to restriction endonucleases except that the specificity would be induced by the experimenter. But, how to accomplish this? Two notions come to mind. The first assumes that, as previously discussed, antipeptide antibodies work via an induced fit mechanism and thus distort the site at which they bind. If one could supply additional energy the protein might hydrolyze at the site of binding. Although possible, this approach seems unlikely to succeed easily. An alternative approach is to make antibodies to intermediates in catalysis to tip the equilibrium in favor of hydrolysis of the peptide bond. The principle, then, is one of immunological catalysis.

Antibodies and enzymes are both proteins whose function depends on their ability to bind specific target molecules. Enzymatic reactions differ from immunological reactions in that the binding of substrate to enzyme leads to chemical catalysis. Enzymes catalyze the hydrolysis of proteins, by combining with the protein in a *transition state* of the reaction. It is generally assumed that an enzymatic reaction is accelerated with respect to the nonenzymatic reaction because of the enzyme's ability to reduce the free energy of the transition state, and thus, the free energy of activation, of the reaction (Jencks, 1975; Pauling, 1948). The enzyme might accomplish this by binding a transition state geometry more strongly than the corresponding substrate(s) or product(s). This means that an enzyme's intrinsic binding energy is much greater than can be measured from the binding of substrates or products. Essentially, the enzyme's binding energy is utilized to perform the chemical reaction (Jencks, 1983).

The basic idea behind immunological catalysis contemplates the design of antibodies of predetermined specificity that will stabilize transition states of peptide bond hydrolysis upon binding to the specified antigen. This should result in a reduction in the activation energy for the hydrolysis reaction, thus meeting a criterion for catalysis. Antibodies which display this property might be obtained by immunization with synthetic peptide analogs that are chemically modified to resemble the bonding characteristics of a substrate undergoing peptide bond hydrolysis—that is, *transition state analogs* of this reaction. The mechanism by which an antibody might catalyze the hydrolysis of a bound substance may be thought of in terms of an "induced fit" model. As loosely bound substrate distorts to conform to the binding geometry of the antibody, stress can be relieved by chemical reorganization of a single amide bond such that this reorganization leads toward hydrolysis of the bond.

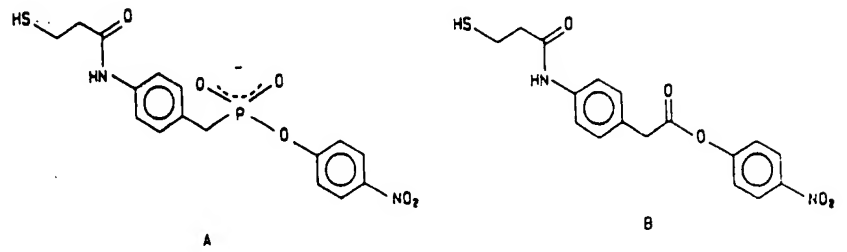

FIG. 9.

Toward the goal of designing immunological catalysts, we have begun to synthesize chemical analogs of polypeptides and depsipeptides which incorporate phosphonamidate or phosphonate moieties at specific sites to mimic the transition state for hydrolysis of the amide or ester bond (A. Tramontano, Lin, Bor-Sheng, and Lerner, unpublished results). These are reasonable candidates for this experiment because it is known that such phosphonamidates are, in fact, transition state analogs in the inhibition of proteolytic enzymes (Bartlett and Marlowe, 1983). Initially, we plan to investigate a system designed for the hydrolysis of a simple *p*-nitrophenyl ester. To illustrate some of the ideas, we can consider some sample compounds which we have prepared. Compound A is being prepared to act as the transition state analog of *p*-nitrophenyl ester (Compound B) in an immunological experiment (Fig. 9). Antibodies generated to Compound A bound to a carrier can be isolated and screened in an assay which tests for catalytic hydrolysis of ester (Compound B). The liberation of the colored *p*-nitrophenolate in this reaction will facilitate the detection of catalytically active antibodies. The following synthesis scheme is being used: diethyl *p*-aminobenzyl phosphonate is condensed with 3,3'-dithiopropionic acid to provide the dimeric amide. This is transesterified at phosphorous to give the di-*p*-nitrophenyl ester, mild base hydrolysis and reduction of the disulfide provides substance A. The ester (Compound B) is prepared by a similar sequence, starting with ethyl *p*-aminophenyl acetate. In the immunological experiment the phosphonate/phosphonamidate may be coupled to carriers through a disulfide bond, though other functional groups may be employed.

Hydrolysis of the amide bond of polypeptides or proteins will require analogs which bear the phosphonamidate moiety (Fig. 10). Methods for the synthesis of these compounds are being explored. Phosphonamidates described for the inhibition of certain proteases (Bartlett and Marlowe, 1983; Jacobsen and Bartlett, 1981) can also be modified for induction of immunological catalysts. Since short poly-

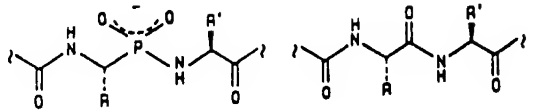

FIG. 10.

peptide chains can induce antibodies which recognize the protein at specific sites, we may expect that if an antibody specified for a transition state analog catalyses the hydrolysis of a short polypeptide chain, it will also catalyze the cleavage of a protein with that particular sequence of amino acids somewhere along its length. The implication of these expectations is that we could confer the activity of certain proteases to immunoglobulins. Furthermore, the antibody's activity may be directed to any site at will by designating the amide bond to be cleaved with the phosphonamidate center in the analog used for immunization. Thus, a method would be available for the selective proteolysis of any protein whose local sequence conforms with that of the polypeptide targeted. The applications of such a method in protein chemistry, biochemistry, and medicine are without limit. For example, instead of engineering the immune system to simply bind to viruses or tumor antigens, we could aim at evoking antibodies which catalyze specific protein cleavages which inactivate viruses or kill cells. In essence, one evokes antibodies which act directly and do not depend on help from accessory factors such as complement, or complex processes like opsinization. Regardless of whether these concepts are correct in detail, one can expect antibodies of predetermined specificity to soon take on roles which may transcend their simple binding functions. (See Note Added in Proof.)

XVII. Antibody Template Directed Organic Synthesis

It is a curious paradox that although almost all biological processes are mediated by proteins, very few proteins are useful as therapeutics. There are, of course, good reasons for this. Proteins are labile, often destroyed by the acid pH of the stomach, unable to cross cell membranes or the blood–brain barrier, etc. Even a lifesaving protein such as insulin could hardly be classified as an ideal drug, in that it requires daily injection and as currently given does not prevent the many complications of diabetes. Also, important peptides such as hypothalamic releasing factors cannot easily be turned into drugs because of their limited ability to be absorbed after ingestion or cross the blood–brain barrier. So, what one really wants is to construct nonpeptidyl organic mimics of proteins and peptides. One knows that this is possible because, for example, the endorphins and the opiates share the same receptor—it is just that when we look at the two structures we do not understand why the receptor "sees" them as mimics of each other. In other words, we do not know the rules by which nonpeptidyl organic compounds mimic proteins. If, however, these rules could be deciphered one would see a new approach to rational design of drugs. In an interesting way, antibodies of predetermined specificity have opened a wedge which could lead to a better understanding of how to make organic mimics of proteins. The way in, of course, is to learn by making organic mimics of antigens. As long as one was dealing at the whole protein level, there was little hope for such design. But, now that the problem can be reduced to only a few amino acids, it is probably fair to say that the problem has moved from impossible to formidable. The process one can use to design organic mimics of proteins is that of antibody template directed organic synthesis. In this process one begins with a monoclonal antibody and a peptide mimic of a protein antigen (i.e., as described above for the influenza system). This is essentially the same as having, in organic chemical terms, a pure host and a pure guest. In practice, each time a chemical change is made in the peptide ligand, its ability to react with the antibody is checked. If the ligand still reacts, the chemical step was permitted and the next step can be taken. If the antibody does not bind the ligand a different modification must be made. By continuing this process, one would hope to evolve a compound which no longer resembles the peptide but still shares immune reactivity. Indeed, nonpeptidyl organic antigens would be useful, but the real goal is to learn the rules for chemical mimicking of proteins. If this could be accomplished, chemicals which mimic the functions of proteins, such as insulins and even intracellular regulators, might be a reality.

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NOTE ADDED IN PROOF. A somewhat different approach to antibody-mediated ester hydrolysis has been taken by Kohen and colleagues (F. Kohen *et al.*, *FEBS Lett.* 111, 427, 1980; *Biochim. Biophys. Acta* 629, 328, 1980). Based on studies which showed that antibodies against haptens may enhance hydrolysis of labile esters, they explored the effect of antibody against 2,4-dinitrophenyl on the hydrolysis of DNP-E-aminocaproyl-umbelliferone. The anti-DNP antibody seemed to induce hydrolysis of an ester which was six atoms removed from the hapten but the reaction was stoichiometric. Such hydrolysis is not truly catalytic however since the product of the reaction may bind as well or better than the ester to the antibody, thus inhibiting further reaction (Kohen *et al.*, 1980). The binding properties of the antibody must, therefore, take into account the characteristic features of the reaction, as enzymes do, for the protein to express a catalytic function. An additional problem is that antibodies are, in fact, capable of hydrolyzing labile esters in a nonspecific manner (L. I. Slobin, *Biochemistry* 5, 2836, 1966). This behavior is not dependent on binding of the ester but may merely reflect the nucleophilic quality of the hydrophilic amino acids on the antibody's surface. Although these studies are of interest, it would seem that a more general approach would be to divert antibodies to the structure containing the bond to be hydrolyzed rather than some distant moiety.

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A Molecular Analysis of the Cytolytic T Lymphocyte Response

STEVEN J. BURAKOFF, OFRA WEINBERGER, ALAN M. KRENSKY, AND CAROL S. REISS

Department of Pediatrics, Harvard Medical School, the Dana-Farber Cancer Institute and the Children's Hospital Medical Center, Boston, Massachusetts

I. Introduction

The cytolytic T lymphocyte (CTL) response has been an area of intense investigation for the past 15 years. Though initially studied for the response to allogeneic major histocompatibility complex (MHC) antigens, the observations of Zinkernagel and Doherty (1974) and of Shearer (1974) have resulted in enormous interest in the CTL response to modified syngeneic MHC antigens.

The availability of a reliable and easy assay for CTL activity, i.e., the chromium release assay developed by Brunner *et al.* (1968), resulted in rapid progress in our understanding of the CTL response. Initially, the specificity of CTL recognition could be explored because of the availability of inbred strains of mice bearing recombinations within the major histocompatibility complex. However, there has always been a major limitation to a better molecular understand-

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